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# Feeding, survival, and reproduction of two populations of *Eurytemora* (Copepoda) exposed to local toxic cyanobacteria

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Abbreviated running title: Effects of cyanobacteria on *Eurytemora*

**Abstract**

Understanding lower food web interactions in the Laurentian Great Lakes can be furthered by experimental comparisons among locations with similar ecological stresses, such as harmful algal blooms. Here we compare responses to toxic cyanobacteria by crustacean copepods of the genus *Eurytemora* from eutrophic coastal regions of Lake Michigan and the Baltic Sea. We measured grazing, survivorship, reproduction, and juvenile (nauplius) size, incubating females in experimental treatments holding good food and mixtures of good food with either cyanobacteria or cyanobacteria filtrate. Animals tested were from Green Bay, Lake Michigan and Gulf of Finland, Baltic Sea. Results showed similarities between copepods in the two study locations; when fed mixtures of cyanobacteria and good food there were no effects in either location on survivorship, grazing rates, or fecundity, but copepods in both sites were most sensitive to good food combined with cyanobacteria filtrate (in absence of cyanobacteria cells). Filtrate exposure significantly reduced grazing by animals in both locations and decreased adult survival and nauplius size in the Baltic experiment, suggesting animals responded to toxins or other compounds entering the water. These responses may be due to direct effects on females or indirect effects from changes in good food quality. Our results also demonstrated a significant trade-off between offspring quantity and quality, being more pronounced when food quality was manipulated by the presence of cyanobacteria cells. These findings further our knowledge of how a widely distributed group like *Eurytemora* can succeed in the face of changing local selection pressures from natural and anthropogenic stressors.

**Key words**

*Microcystis*, *Nodularia*, HAB, feeding, survival, cyanotoxin

## Introduction

The expansion and persistence of cyanobacterial blooms are increasing globally due to human-induced eutrophication (Paerl and Otten, 2013). Such eutrophication of coastal areas is occurring in diverse locations including the Laurentian Great Lakes (Binding et al., 2015), Lake Baikal (Timoshkin et al., 2016), the Baltic Sea (Andersen et al., 2017) and estuaries worldwide (Bricker et al., 2008). Climate change, acting via global warming and changes in factors such as precipitation, salinity and pH, further promote the development and frequency of cyanobacteria mass-occurrences (O'Neil et al., 2012). Effects of cyanobacterial blooms on lower food web interactions are especially important due to the intimate connections between zooplankton and phytoplankton (Ger et al., 2014). Cyanobacteria have traditionally been considered low quality food for zooplankton due to low manageability, minor nutritional quality, and toxin content (Porter and Orcutt, 1980; Lampert, 1987; DeMott and Moxter, 1991). A large meta-analysis of published studies on the effects of cyanobacteria on zooplankton population growth and survivorship reaffirmed the importance of nutritional effects, but also highlighted the lack of work examining how fitness of copepods is affected by cyanobacteria (Wilson et al., 2006). More recent work has focused on key traits that zooplankton rely on to improve their fitness, including physiological tolerance (such as detoxification pathways), and avoidance of poor quality food by selective feeding behaviors. Such characteristics allow calanoid copepods to co-exist with toxic cyanobacteria, and even become more tolerant of blooms over time through local selection and adaptation (Ger et al., 2014, 2016).

Work on cyanobacteria-zooplankton interactions studying the calanoid copepod *Eurytemora affinis* and toxic algae indicate contrasting results (Ger et al., 2016). Previous studies have shown low egg production and survival when copepods are fed toxic cyanobacteria (Koski et al., 1999; Kozlowsky-Suzuki et al., 2003). However, Schmidt and

77 Jónasdóttir (1997) observed that while the cyanobacteria *Microcystis aeruginosa* was
78 inadequate as a single food source due to poor nutritional quality, it was beneficial in small
79 doses as a supplement to the main diatom food source *Thalassiosira weissflogii*. Vehmaa et
80 al. (2013) also observed a positive effect of the cyanobacterium *Nodularia spumigena* on
81 reproduction of copepods when provided with a mixed diet. The main negative effects of
82 toxic cyanobacteria on zooplankton are expected to arise from direct dietary exposure, such
83 as ingestion of toxic cells and poor nutritional impacts (Ger et al., 2014). Dissolved toxins
84 derived from cyanobacteria during bloom conditions, such as the hepatotoxin microcystin
85 produced by *M. aeruginosa* and many other species, are not considered a main threat for
86 copepods like *E. affinis* because typical field concentrations of microcystin are orders of
87 magnitude below lab-determined lethal levels (Ger et al., 2009, 2016). Additional studies
88 with *E. affinis* also suggest that toxicity may not be the most important factor governing
89 zooplankton-cyanobacteria interactions. For instance, when availability of good food is
90 reduced *Eurytemora* are able to feed more readily on toxic *Nodularia spumigena* despite high
91 amounts of dissolved toxins (Gorokhova and Engström-Öst, 2009). However, in addition to
92 toxins, cyanobacteria produce other compounds during blooms that may have more direct
93 negative effects on zooplankton (Ger et al., 2016). Also, morphological traits such as
94 filament or colony size, and history of co-occurrence are important factors, as smaller
95 filaments and long co-evolutionary history may favor feeding on toxic strains when good
96 food is scarce. As predicted by optimal foraging theory, selective feeders like copepods
97 should discriminate more strongly against low-quality algae when food concentrations are
98 high (cf., DeMott 1989). These findings stress the need to further investigate effects of toxic
99 algae on copepods, such as those in the *Eurytemora* group.
100 *Eurytemora affinis* is a common species in coastal areas, estuaries, and marshes of the
101 Northern Hemisphere where it is a major link in the lower food web, feeding on both algae

and toxic cyanobacteria (Engström et al., 2000) and providing food for young fish (Ger et al., 2010). It also expanded into freshwater habitats such as the Laurentian Great Lakes during the middle of the 20$^{th}$ century (Lee, 1999), and recent studies even consider clades in the Great Lakes a separate species (*E. carolleeae*; Alekseev and Souissi, 2011). Lee et al. (2013) demonstrated that high food availability was likely a central factor allowing *E. affinis* to spread from brackish water to freshwater areas, and research in the St. Lawrence estuary where both invasive and non-invasive clades coexist indicate that feeding behaviors differ among the clades (Favier and Winkler, 2014; Cabrol et al., 2015). Studying the feeding ecology of invasive copepods like *Eurytemora* can provide insight into how zooplankton might respond to new feeding conditions following range expansion into novel habitats.

Local adaptation of *Eurytemora* to feeding on phytoplankton in eutrophic regions involves responses to toxic cyanobacteria blooms, a major result of eutrophication. Both study areas in the current work, Green Bay (Lake Michigan, USA) and the Gulf of Finland (Baltic Sea), suffer from annual cyanobacterial mass-occurrences. The main source of toxic blooms in the Gulf of Finland is a filamentous species, *Nodularia spumigena*, producing a hepatotoxin called nodularin (Suikkanen et al., 2010). In Green Bay the dominant cyanobacteria species is now *Microcystis aeruginosa*, which can grow either as solitary cells or colonies. It is now common in many Great Lakes regions, producing microcystin, a hepatotoxin closely related to nodularin (Mur et al., 1999; De Stasio et al., 2014). Comparing the responses of *Eurytemora* to these local cyanobacteria and their toxins can help determine potential changes in food web interactions in the face of increasing eutrophication.

The aim of this study was to compare the effects of toxic cyanobacteria on *Eurytemora* from Green Bay and the Baltic Sea using a comparable experimental design in parallel experiments. We examined survivorship, feeding, egg production, and size of nauplii produced by animals from the two sites in response to manipulation of representative species

of toxic cyanobacteria, dissolved substances produced by the cyanobacteria and high quality algal food similar to local food resources. Given the known sensitivity of *Eurytemora* to cyanobacteria in the native range we hypothesized that feeding, survivorship, and egg production would be negatively affected by the presence of toxic cyanobacteria. We also expected there would be separate effects of extracellular toxins and other metabolites released by cyanobacteria distinguishable from nutritional effects due directly to feeding on the cyanobacteria cells. Furthermore, because of the relatively recent invasion of the Great Lakes by *Eurytemora* and the short time period of exposure to freshwater cyanobacteria species, we expected animals from Green Bay to be more sensitive to toxic cyanobacteria due to the briefer history of selection under local environmental conditions.

## Methods

### *Study sites*

Green Bay, Lake Michigan is the largest freshwater estuary of the Laurentian Great Lakes, and exhibits high primary productivity and strong physical and trophic gradients (Richman et al., 1984; De Stasio et al., 2008). In the shallow southern region there is no persistent stratification due to frequent mixing (Qualls et al., 2007). The southern bay is also influenced by high nutrient and sediment loading entering from the lower Fox River resulting in considerable primary productivity throughout the summer months (Stoermer, 1978; LaBuhn and Klump, 2016). As a result, Green Bay suffers from nuisance phytoplankton blooms. In addition, the phytoplankton community in the bay has shifted to increased dominance by cyanobacteria (*Anabaena, Aphanizomenon, Microcystis*) following a zebra mussel invasion (De Stasio et al., 2014). Concentrations of the hepatotoxin microcystin typically range from 0.3 to 1.7 µg/L (McDermott et al., 1995; B. De Stasio, unpublished data) in the southern bay.

151 The Gulf of Finland is the most eastern region of the Baltic Sea and is fairly shallow
152 (average depth 38 m), with limited vertical mixing due to slow water exchange. This creates
153 horizontal gradients of salinity (surface: 0.2 - 5.8) and temperature (summer SST: 15 - 17 ºC)
154 (Myrberg et al., 2006; Suikkanen et al., 2013). The Gulf of Finland is predominantly nitrogen
155 limited during the productive season (spring and late summer), which allows intensive
156 nuisance blooms, mainly of non-toxic N-fixing *Aphanizomenon* sp. and hepatotoxic
157 *Nodularia spumigena.* Concentrations of nodularin, the main toxin produced by *Nodularia*,
158 typically vary between 0.5 and 2.6 µg/L (Kankaanpää et al., 2001). Cyanobacteria blooms in
159 the Baltic Sea have been common since at least the 19$^{th}$ century, but have increased due to
160 human-induced impacts (Bianchi et al., 2000).
161
162 *Study species*
163 *Eurytemora affinis* is native to northern temperate coastal areas around the world but was
164 introduced to North American freshwater systems and first documented in 1880 (Lee, 1999).
165 It was later reported in Lake Michigan by Robertson (1966), and since the late 1960s the
166 species is found in both littoral areas and pelagic plankton communities in Green Bay
167 (Gannon, 1974). The species complex has spread multiple times independently from brackish
168 water to freshwater, and established clades in different parts of the world due to its ability to
169 adapt locally (Dodson et al., 2010). There are at least six recognized clades of *E. affinis*; one
170 clade in Asia, one in Europe, and four in America (Lee, 2000), with recent studies indicating
171 that North American clades represent a separate species (*E. carolleeae*; Alekseev and Souissi,
172 2011; Vasquez et al., 2016). *Eurytemora* in Lake Michigan waters is most common between
173 July and November, being rare in winter and spring (Torke, 2001). The work by Vasquez et
174 al. (2016) has shown that all previous samples from the Great Lakes (including from Green
175 Bay) were *E. carolleeae* and we assume that all animals employed in our Green Bay

176 experiment were *E. carolleeae* resulting from the freshwater invasion of the Great Lakes
177 during the late 1950s (Lee, 1999; Lee et al., 2013).

178 In the Baltic Sea *E. affinis* has its abundance peak in June and July (Viitasalo, 1992)
179 and is native to the system (Lee, 1999). *E. affinis* occurs both in pelagic and coastal areas in
180 the Baltic Sea (Viitasalo, 1992). There is also evidence of recent invasions into the Baltic Sea
181 by clades from North America (Sukhikh et al., 2013). It is unknown if the *Eurytemora* we
182 collected at the Tvärminne Zoological Station were exclusively *E. affinis*, but because *E.*
183 *carolleeae* were found in other areas of the Gulf of Finland we refer to animals used in our
184 Baltic Sea experiment as *Eurytemora* sp.

Insert Table 1 near here

185 *Experimental Design*

186 We compared the response of *Eurytemora* to toxic cyanobacteria by conducting parallel
187 experiments with a common design but using animals from either the Baltic Sea or Green
188 Bay (Table 1). Food treatments were created so that all animals received the same quantity
189 (500 µg C/L) of a food source representative of local algae and of sufficient quality to
190 provide good growth and survivorship conditions during the experiment. This concentration
191 was intended to keep food resources above limiting levels, resulting in maximum ingestion
192 rates on good food. In the "good food" treatment (GF) this was the only source of nutrition.
193 The "cyanobacteria" treatment (CYAN) contained the good food and additional cultured
194 toxic cyanobacteria to achieve external toxin concentrations similar to local conditions. In the
195 Baltic experiment this amounted to adding 100 µg C/L of *Nodularia spumigena* and for
196 Green Bay 50 µg C/L of *Microcystis aeruginosa* (Table 1; see below for cyanobacteria strain
197 information). The third treatment received the same amount of good food plus a volume of
198 filtrate from the cyanobacteria source equivalent to the volume of cyanobacteria solution
199 added in the CYAN treatment. Consequently the CYAN and FILT treatments differed only

in the presence or absence of cyanobacteria cells, and the GF and FILT treatments differed only in extracellular components added with the cyanobacteria cells.

*Strain cultivation*

Four different phytoplankton species were used as food sources in the experiments: the cryptophyte *Rhodomonas salina* and the toxic cyanobacterium *Nodularia spumigena* in the Baltic Sea, and the chlorophyte *Scenedesmus quadricauda* and the toxic cyanobacterium *Microcystis aeruginosa* in Green Bay. In the Baltic Sea experiment the *Rhodomonas salina* culture (Cryptophyceae; 07B6; obtained from Dr. Anke Kremp, Finnish Environment Institute) was grown using f/2 medium at 18°C in ~10 µmol photons/$m^2$/s with a 16 : 8 h light : dark regime and a salinity of 6‰ in aged seawater. The *Nodularia spumigena* culture (strain AV1, a potent nodularin producer) was obtained from Prof. Kaarina Sivonen, University of Helsinki and grown in Z8-N nutrient solution (Sivonen et al., 1989) with modified salinity (6‰) at 18°C in a 16 : 8 h light : dark regime. Cell counts for initial culture concentrations in the Baltic Sea experiments were performed using the Ütermöhl method with transect counts using an eyepiece micrometer. *Nodularia* filament lengths (64.04 ± 5.86 µm, mean ± SE) were converted into cell density, while individual cell counts were determined directly for *Rhodomonas* (length: 19.8 ± 0.61 µm). To determine food concentration in µg C/L, cell densities were converted with biovolume estimates and carbon conversion factors (Montagnes et al., 1994; Olenina et al., 2006). Appropriate volumes of algae and cyanobacteria from exponentially growing cultures were added to each treatment bottle with FSW, filtered seawater (0.2 µm pore size, Sartobran 300 filters, Sartorius Stedim Biotech GmbH. Göttingen, Germany) to obtain desired initial concentrations of food (used within ~24h). Filtrate from the *Nodularia* cultures for use in the experiment was obtained by filtering (GF/C, Whatman, nominal pore size 1.2 µm) the appropriate amount of culture.

For Green Bay experiments both cultures were grown in freshwater media and were initiated from batch cultures. The *S. quadricauda* culture (Carolina Biological Supply, Burlington, NC) received Bristols solution made with Milli-Q water (125 mL/L) to optimize growth rates. The toxic *Microcystis aeruginosa* strain (PCC 7820; Pasteur Institute, Paris, France) was grown in Cyanobacteria BG-11 Freshwater Solution (C3061; Sigma Chemical, St. Louis, MO) at a concentration of 20 mL/L in Milli-Q water. The *S. quadricauda* culture was kept gently aerated and maintained under exponential growth conditions at room temperature (20°C) in direct sunlight near a window. The *M. aeruginosa* also was kept at room temperature in moderate, but indirect, sunlight and stirred gently on a shaker table. *Microcystis* grew in this culture as a mixture of single and bicells. Prior to use in the experiment each culture was centrifuged (3000 rpm, 30 min) to separate cells from growth medium, which can be toxic to animals in high concentrations. Pelleted cells were resuspended in filtered lake water (GF/C, Whatman) and then enumerated for use in the experiment. To determine the amount of food to be added in the treatments, cell densities of algal and cyanobacteria cultures were estimated with a hemocytometer counting chamber and converted to µg C/L based on mean cell size (*Scenedesmus*: 12.7 um, *Microcystis*: 4.1 um) and biovolume estimates obtained at 400X and a carbon content conversion factor of 0.2 pg C/$\mu m^3$ (Reynolds, 1984; Rocha and Duncan, 1985). Filtrate from *Microcystis* cultures for use in the experiment was obtained by filtering (GF/C, Whatman) the appropriate amount of resuspended culture.

*Field sampling*

In the Baltic Sea experiment, seawater was collected at 5 m depth off the Tvärminne Zoological Station at the entrance to the Gulf of Finland (N 59.8556°, E 23.2617°) using a Limnos water sampler (Hydrobios, Germany). Immediately after collection, the seawater

sample was filtered (0.2 µm pore size, Sartobran 300 filters) and allocated into treatment bottles, stored at 17°C, and used within 24 h of preparation.

*Eurytemora sp.* was sampled over a three-day period (3-6 August 2012) from 25 m depth with four vertical tows, using a 200-µm mesh net (0.485 m diameter) with cod-end collection cup. Samples were gently transferred to a 40-L container with seawater from below the thermocline, and immediately transported to a temperature climate chamber with 16 : 8 h light : dark cycle. Sorting of tow samples took place upon return to the laboratory and desired number of adults were placed in treatment bottles with filtered seawater, inoculated with the treatment algal conditions and stored at 17°C.

Surface water was collected from Green Bay at Little Sturgeon Bay, a small embayment along the southeast shore of the bay (N 44.8452º, W 87.5584º), and transferred via carboy to the lab where it was double filtered (Whatman #1 qualitative filter followed by Whatman GF/C filter). Filtered water was stored at room temperature in the laboratory and used within ~24h. Animals were collected between sunset and midnight on 1 October 2013 by horizontal tows between the surface and ~3 m depth near a boat dock, using a 250-µm mesh net (0.5 m diameter) with cod-end collection cup. Samples were transferred to 6-L buckets containing Little Sturgeon Bay water, transported on ice to the lab, and stored at 17ºC with 16 : 8 h light : dark cycle. *Eurytemora* were transferred individually to beakers of filtered lake water containing treatment food conditions.

*Baltic experiments*

*Grazing* - Copepods were subjected to the three treatments, consisting of different mixtures of laboratory cultures of *Nodularia* and *Rhodomonas* (Table 1): 1) Good Food (GF) consisting of only *Rhodomonas*, 2) Cyanobacteria addition (CYAN) with *Rhodomonas* and *Nodularia*, 3) Filtrate addition (FILT) containing *Rhodomonas* and filtrate from the

*Nodularia* culture (corresponding to the volume of *Nodularia* added in CYAN). Volumes of algae and cyanobacteria cultures added to the treatments were determined through cell counts of each culture as described above under *Strain cultivation*.

Adult *Eurytemora* sp. females carrying egg sacs were sorted from samples and then acclimated in each treatment condition for 60 h. Treatment solutions were made fresh and replaced daily. Following the acclimation period male *Eurytemora* sp. were sorted from fresh samples and added to bottles with the females that had dropped their egg sacs during the acclimation period. This ensured that new eggs were produced by females acclimated to each treatment condition. Each treatment had three replicates with *Eurytemora* sp. (12 females and 3 males per bottle) and three control replicates without animals, carried out in 1.2 L Pyrex glass flasks with a screw cap. This number of animals ensured sufficient feeding to allow measurement of grazing rates without excessive depletion of food. Males were included to fertilize females so reproduction rate and offspring size could be determined. Closed bottles were incubated in a 17°C climate chamber and gently mixed by inversion twice during the experiment to reduce settling of food.

Replicate samples of initial conditions were collected from each bottle for chlorophyll *a*, cell counts and toxin concentration (see below) before addition of copepods. The experiment was run for 24 h, after which final samples were collected. Container contents were then gently filtered through a 63-µm mesh cup to check for adult survivorship, number of females carrying egg sacs, and nauplii produced. Changes in chlorophyll concentration during the experiment were used to determine ingestion rates (µg C/individual/h) according to the standard procedures of Frost (1972) and assuming a 50:1 carbon conversion from chlorophyll (Reynolds 1984).

*Reproduction* - Females carrying egg sacs after the completion of the grazing experiment (GF:23, CYAN:24, FILT:21) were transferred into individual wells of 12-well tissue culture

plates containing the same treatment solution. Tray contents were checked daily and after all eggs in a plate had hatched, acid Lugol's solution was added to preserve nauplii for later counting and measurement. Individual females were assessed for number of eggs produced to determine egg ratios. Fecundity was estimated as egg production rate (eggs/female/day) by dividing egg ratios by egg development time (in days) according to temperature of the experiment using the relationship from Andersen and Nielsen (1997). Development time was 1.92 days at the experimental temperature of 17°C. Lengths of first stage nauplii from each replicate (10 minimum) were measured at 100 X magnification under an inverted microscope with an eyepiece micrometer.

*Green Bay experiments*

*Grazing* - Food manipulation in the Green Bay experiment included analogous treatment conditions as employed in the Baltic Sea experiment but using species more representative of local conditions (Table 1): 1) Good food (GF) consisting of *Scenedesmus*, 2) Cyanobacteria addition (CYAN) including *Scenedesmus* and *Microcystis*, and 3) Cyanobacteria culture filtrate addition (FILT) with *Scenedesmus* and filtrate corresponding to the same volume of *Microcystis* culture added in CYAN. *Scenedesmus* concentration added was kept constant in all treatments and equal to that used in the Baltic Sea experiment (500 μg C/L). Volume of *Microcystis* added was intended to achieve similar toxin concentrations as in the Baltic Sea experiments, resulting in an increased carbon content of 10% compared to that from *Scenedesmus*.

Female *E. carolleeae* carrying egg sacs were acclimated to the experimental treatment conditions for 41 h. Females without eggs sacs at the end of the acclimation period were moved to triplicate beakers (250 mL) containing fresh treatment conditions. Triplicate beakers without animals served as controls. Each beaker with *Eurytemora* contained 12-14

females to achieve measurable feeding rates but not deplete food resources during the experiment. Beakers were held in a 15°C incubator with no light and covered with a sheet of plexiglass to reduce evaporation. Beakers were gently stirred twice during the experiment to reduce settling of algae. During the 22-h grazing experiment survivorship was also recorded. At the end of the grazing experiment, all copepods were removed by gently filtering beaker contents through a mesh cup (128-µm mesh), counting animals and preserving them in formalin (4% buffered). Initial and final samples were taken to assess chlorophyll *a*, cell densities, and toxin concentrations. Cell densities were determined using the same counting procedures employed for quantifying cell cultures and conversion to carbon units (see *Strain Cultivation*). Ingestion rates (µg C/individual/h) were calculated according to the standard procedures of Frost (1972) and carbon conversion as employed in the Baltic experiment.

A separate experiment to determine background starvation rates of *Eurytemora* from Green Bay was conducted along with the grazing experiment. Twelve female *E. carolleeae* previously acclimated to GF conditions as above were placed into separate wells of a tissue culture plate holding 4.5 mL of filtered water (Whatman GF/C). Animals were held at 20°C and checked at 12 h intervals for five days. Water was replaced with freshly filtered water and fraction of females alive was determined at each time point.

*Reproduction* - Fifty females with egg sacs were placed in 250-mL beakers containing the same three treatment conditions as above and acclimated for 40 h at 15°C in constant dark. At the end of the acclimation period females that were not carrying egg sacs were combined in new beakers with males (mean of 7 males per beaker). Three days after the start of the reproduction experiment, females carrying egg sacs (GF:10, CYAN:7, FILT:18) were placed in separate covered petri dishes (35 mm diameter) with fresh treatment food solutions changed daily until all eggs hatched. Individual females were assessed for number of eggs

produced to determine egg ratios. Fecundity was estimated as egg production rate (eggs/female/day) by dividing egg ratios by egg development time (in days) as described above for the Baltic Sea experiment. For the GB experiment, development time was 2.19 days at 15°C. Hatched nauplii were counted daily for each female. After all nauplii hatched, the sample was preserved with acid Lugol's solution for later analysis. Nauplius length measurements were conducted at 100 X magnification on a inverted microscope with an eyepiece micrometer.

*Analytical procedures*

Samples for chlorophyll analysis were filtered onto GF/C Whatman filters and frozen at -20ºC until measurement. For the Baltic Sea experiments, samples were extracted in ethanol according to Jespersen and Christoffersen (1987) and analyzed using a Shimadzu UV-2501 PC spectrophotometer. In the Green Bay experiment samples were extracted using alkalized acetone according to Wetzel and Likens (1991) and analyzed using a scanning spectrophotometer (Cary Model 50) with 50 mm path-length cuvette.

Samples for extracellular toxins in filtrates were collected from water passed through a GF/C Whatman filter and stored at -20ºC. Toxin concentrations for both experiments were analyzed by ELISA, using a microcystin plate kit (EnviroLogix, Portland, ME, USA), according to standard kit instructions. This ELISA kit measures both microcystin and nodularin (cf. Gorokhova and Engström-Öst, 2009); consequently, data for both experiments are reported as microcystin-LR equivalents. Each experiment only contained one kind of toxic algae, so ELISA data represent concentrations of toxin specific to each type of cyanobacteria employed (i.e., nodularin or microcystin).

*Statistical analyses*

374 Data distributions were examined for normality prior to statistical analysis with the
375 Paleontological Statistics (PAST) software package (Hammer et al., 2001). Transformations
376 were successfully applied to some data to meet normality expectations; percentage data were
377 arcsine transformed before analysis whereas LOG or square root transformations were
378 employed for others, followed by back-transformation before reporting. Analysis of Variance
379 (ANOVA) and Tukey-Kramer multiple comparison tests were used when data met parametric
380 considerations. If data were not normally distributed following transformation original data
381 were tested with non-parametric exact permutation tests (9,999 permutations) or Kruskal-
382 Wallis and Mann-Whitney tests with Bonferroni corrections. Relationships of nauplius size
383 and fecundity were examined with linear regression analysis, followed by a Generalized
384 Linear model using the *G* statistic test of slopes equaling zero. Significance was assumed for
385 all tests if *p* values were 0.05 or smaller.

386

387 **Results**

388 As intended, our manipulations of diets exposed animals from both populations to
389 similar amounts of extracellular toxins (Table 1). There was no significant difference in toxin
390 levels, all measured as microcystin-LR, between CYAN or FILT treatments in the Baltic
391 experiment or the Green Bay FILT treatment. Concentrations of nodularin in the Baltic Sea
392 experiment were approximately 0.75 µg/L of microcystin-LR equivalents, while mean
393 microcystin-LR toxin level in the GB FILT treatment was 0.80 µg/L. Toxin in the Green Bay
394 CYAN treatment was slightly lower at 0.52 µg/L, and significantly different than in the other
395 CYAN or FILT treatments (Table 1). Toxin levels in the GF treatments were below detection
396 limits in both experiments, and consequently there was an overall significant effect of
397 treatment on toxin concentrations ($F_{3,14}$=12.08, $p$=0.00004).

398 *Baltic Sea Experiments*

Insert Figure 1 near here

399 Survivorship varied among feeding treatments in the Baltic Sea experiment (Fig. 1A).
400 Survival was high for copepods that fed on the algae *Rhodomonas* in the GF treatment (mean
401 =94.2%). Supplementing food with the cyanobacteria *Nodularia* in the CYAN treatment did
402 not significantly alter survivorship ($Q$=0.29, df=6, $p$=0.978) and the 95% confidence interval
403 of both GF and CYAN include 100% survivorship. Animals in the FILT treatment, which
404 were fed good food but also exposed to cyanobacterium culture filtrate, had significantly
405 lower survivorship compared to animals in GF or CYAN treatments with an average of 40%
406 of the animals surviving (GF comparison: $Q$=4.95, $p$=0.03; CYAN comparison: $Q$=5.24,
407 $p$=0.02). This survivorship rate is lower than the expected rate of 87% following starvation
408 conditions based on previously published data for this population (Koski et al., 1999).

409 Animals in the Baltic Sea experiment seemed varied their feeding depending on the
410 food treatment (Fig. 1B). Mean ingestion rate was highest when feeding on only good food
411 (GF mean =0.53 µg C/copepod/h). Mean rate for the CYAN treatment was lower than for
412 GF, and animals in the FILT treatment essentially did not feed (Fig. 1B). There was no
413 significant difference between the GF and CYAN ingestion rates (*t*-test exact permutation
414 test: $p$=0.30) but ingestion in FILT was significantly lower than in GF ($p$=0.05).

415 Manipulation of feeding conditions did not significantly affect reproductive output of
416 *Eurytemora* sp. from the Baltic Sea (Fig. 1C; $F_{2,64}$=1.41, $p$=0.252). Females in the GF
417 treatment produced an average of 5.55 eggs/female/day during the experiment. Fecundity of
418 animals in the CYAN treatment appeared decreased compared to the other two treatments,
419 but variability within treatments was high leading to non-significant differences overall.

420 Mean size of first stage nauplii was significantly decreased by the feeding treatments
421 (Fig. 1D; $F_{2,304}$=32.75, $p$<0.0001). Nauplii in the GF treatment averaged 0.145 mm and were
422 significantly larger than those in CYAN (mean=0.137 mm; $Q$=4.51, $p$=0.004). The smallest

nauplii occurred in the FILT treatment with a mean of 0.125 mm, significantly smaller than the other treatments (GF comparison: $Q$=11.88, $p$<0.0001; CYAN comparison: $Q$=7.36, $p$<0.0001).

Insert Figure 2 near here

*Green Bay Experiments*

*Eurytemora* from Green Bay survived well in the feeding experiments, with no significant differences among the treatments (Fig. 2A; $F_{2,6}$=1.34, $p$=0.331). Over 94.2% of animals survived on average in the GF treatment, while survival in the CYAN treatment exhibited a mean of 89.4%. In the FILT treatment animals survived at a rate of over 92.7%. In comparison to these values, animals in the separate starvation experiment exhibited a mean survival rate of 85.1% (SE= 0.9%) for an equal period of time. Overall, starvation rates were linearly related to time with a mortality rate of 0.68% per hour (SE = 0.06%; $R^2$ = 0.977, $t$= -11.25, $p$ = 0.002).

Animals ingested significantly different amounts of carbon depending on the treatment manipulations (Fig. 2B; $F_{2,6}$=13.5, $p$=0.006). Mean ingestion in GF conditions was 0.79 µg C/copepod/h, not significantly different than the mean of 1.08 in the CYAN treatment ($Q$=4.91, $p$=0.312). When exposed to filtrate from the cyanobacterium culture (FILT) copepods showed significantly reduced ingestion rates with a mean of 0.30 µg C/copepod/h ($p$<0.05 for both comparisons).

Similar to results from the Baltic Sea experiment, *E. carolleeae* from Green Bay did not exhibit different egg production based on feeding treatments (Fig. 2C; $F_{2,32}$=0.62, $p$=0.546). Mean fecundity values ranged from 5.02 (FILT) to 6.32 eggs/female/day (CYAN) with animals in the GF treatment producing an average of 5.15 eggs/female/day. These fecundity values were essentially the same as those obtained in the Baltic Sea experiment for GF and FILT treatments (see Fig. 1C).

In contrast to the Baltic Sea results, nauplius size was not affected significantly in the Green Bay experiment (Fig. 2D; $F_{2,113}$=0.24, $p$=0.791). Mean nauplius length was approximately 0.09 mm for all feeding treatments. Interestingly, mean length of nauplii in the Green Bay GF (Fig. 2D; 0.090 mm) was significantly smaller than mean length in GF of the Baltic Sea experiment (Fig. 1D; 0.145 mm; $t$=31.738, df=172, $p$<0.0001 ). These differences in nauplius length in GF reflected the trend for adult body length between the two populations. Adult female *E. carolleeae* from Green Bay were significantly smaller ($t$=13.16, df=75, $p$<0.0001) than the *Eurytemora* sp. used in the Baltic Sea experiment (mean prosome length +/- 1 SE: GB =0.780 +/- 0.008 mm; Baltic= 0.950 +/- 0.010 mm).

*Comparison of nauplius length and Eurytemora fecundity*

Significant negative relationships between average size of nauplii and number of eggs produced occurred in both experiments (Fig. 3). Slope of the linear relationship for Baltic Sea copepods in GF conditions was -0.0015 (SE=0.0004) and was significantly less than zero ($G$=12.54, $p$=0.0004). Slope for animals fed *Nodularia* in the CYAN treatment was even more negative (mean= -0.0023, SE= 0.0009) and was also significantly lower than zero ($G$=6.13, $p$=0.013). In the FILT conditions there was no significant relationship (Fig. 3A). A similar result was obtained in the Green Bay experiment, but only the relationship for the CYAN treatment was significant (Fig. 3B; $G$=8.04 $p$=0.004) with a slope of -0.001 (SE=0.0004).

Insert Figure 3 near here

## Discussion

There has been conflicting evidence reported in studies of copepod feeding on cyanobacteria concerning the separate effects of dissolved toxins and other compounds produced during bloom conditions compared to nutritional impacts of ingested cells. We have shown that

substances dissolved in filtrates negatively affected the copepod *Eurytemora* from both the Baltic Sea and Green Bay. Ingestion rates for both populations were significantly reduced in the FILT treatment, where filtrate was added to good food, compared to feeding just on good food. In addition, these effects were reduced if animals were fed a mixed diet of good food and cyanobacteria. In addition, both populations exhibited a trade-off between offspring size and reproductive rates when feeding on the mixed diets of cyanobacteria and good food.

Animals from the two locations responded differently in terms of other traits measured. *Eurytemora* sp. from the Baltic Sea exhibited decreased survivorship and smaller nauplius length in the FILT treatment whereas animals from Green Bay did not. Overall, *Eurytemora carolleeae* from Green Bay was not more sensitive to the effects of cyanobacteria than *Eurytemora* sp. from the Baltic Sea despite having invaded this freshwater estuary relatively recently.

*Grazing*

Ingestion rates were significantly reduced for both populations of *Eurytemora* in the FILT treatments compared to good food treatments (Fig. 1B and Fig. 2B). In the Green Bay feeding experiment, ingestion by *E. carolleeae* was also significantly lower in FILT compared with the treatment with good food and cyanobacteria cells together (CYAN). While copepods in the FILT treatment had only the good food available as a source of nutrition, they were simultaneously exposed to cell-free filtrate of toxic cyanobacteria. Although there were no differences in quantity of good food available in the FILT treatments, *Eurytemora* could have been indirectly affected via changes in good food cell quality caused by the cyanobacterial filtrate, thereby leading to decreased ingestion by the copepod. A similar phenomenon has been documented in other studies. Cell-free filtrates of the haptophyte *Prymnesium* were shown to indirectly affect ingestion and growth of the rotifer *Brachionus* via negative effects of filtrate on the cryptophyte *Rhodomonas* (Barreiro et al., 2005). Similarly, Suikkanen et al.

(2006) showed that the algae *Rhodomonas* was affected by *Nodularia* filtrate, but not specifically by purified nodularin.

Another possible explanation for the decreased ingestion rates in FILT is that *Eurytemora* changed its feeding behavior due to toxins or other secondary metabolites released from cyanobacteria cells into the filtrate solution (Barreiro et al., 2005; Sopanen et al., 2008). This is a plausible explanation as survivorship and nauplius size also were decreased in the Baltic Sea FILT treatment. Similarly, female *Acartia* sp. showed decreased condition factors during post-bloom conditions in the Baltic Sea (Engström-Öst et al., 2015), suggesting that disrupting blooms (comparable to what occurred in our filtrate preparation) can be harmful. Many copepods are considered selective feeders and able to continue feeding on good food in the presence of toxins (DeMott and Moxter, 1991; Ger et al., 2016), but other crustacean zooplankton often stop feeding (Ger et al., 2014). While some populations of *Eurytemora* have been shown to tolerate microcystin (Ger et al., 2009, 2010, 2014) and perhaps the closely related nodularin (Engström-Öst et al., 2002), it does not mean that feeding in our experiments decreased due to toxin exposure, per se. Other secondary metabolites may be likely to have caused the effects observed in the FILT treatments. Even though cyanobacteria toxins are studied a great deal (recent reviews by Rastogi et al., 2014; Pearson et al., 2016), other metabolites have attracted far less attention, and many of them are not even known to science. Rapid progress is being made on characterizing these substances (Mazard et al., 2016), but this area still needs further research.

In the Green Bay experiment, although the copepods did feed quite actively on *Microcystis* in the CYAN treatment (approximately 40% of total ingestion; data not shown), they showed high survival and reproduction in this particular treatment, suggesting they benefitted from this food mixture despite the presence of toxins and other extracellular components in solution (cf. Vehmaa et al., 2013; Hogfors et al., 2014). Similarly, in the

Baltic Sea experiment there was no significant difference between ingestion rates of *Eurytemora* sp. provided with just good food or with the mixed diet. This finding that *Eurytemora* sp. can utilize *Nodularia* is consistent with previous studies showing that Baltic *Eurytemora* actively ingests *N. spumigena* (Engström-Öst et al., 2002, 2011) as well as studies showing that calanoid copepods can manipulate and feed on filaments that are relatively straight (Vanderploeg et al., 1998), like the *Nodularia* used in our experiment.

*Survival*

*Eurytemora* from the two populations exhibited different survivorship responses to FILT treatments. The Baltic Sea population survived well when feeding on the mixed diet of *Rhodomonas* and *Nodularia* (Fig. 1A). This result is supported by Reinikainen et al. (2002) who also found negligible effects of *Nodularia* or nodularin on *E. affinis* survival. On the other hand, in the Baltic experiment, *Eurytemora* survivorship was significantly reduced in the treatment with *Rhodomonas* and *Nodularia* filtrate. This survivorship rate was much lower than rates expected based on starvation conditions previously determined for this same population (Koski et al. 1999) indicating that something in the filtrate caused increased mortality. This result is consistent with data presented in Sopanen et al. (2008) where stronger negative effects were observed in filtrate than in mixtures when 'good' and 'bad' food were present. Given the likelihood that our *Nodularia* filaments were disrupted following filtration and intracellular contents entered the filtrate treatment, *Eurytemora* may have responded strongly (i.e., with increased mortality) to either released toxin or other metabolites (cf. Sopanen et al., 2008). Decreased condition of the animals most likely also had consequences for feeding rates, as evidenced by decreased ingestion in the filtrate treatment.

Survivorship in the Green Bay experiment was not significantly altered between the treatments. This suggests that this population of *Eurytemora* is tolerant to toxic

546 cyanobacteria, consistent with an earlier study showing this population feeds well on late
547 summer phytoplankton from this location (Richman et al., 1980). Ger et al. (2010) also found
548 no changes in mortality of *Eurytemora* exposed to toxic *Microcystis*, consistent with our
549 results. This tolerance to toxic algae is likely related to previous exposure to *Microcystis*,
550 possibly leading to decreased sensitivity to dissolved microcystin (Sarnelle and Wilson, 2005;
551 Ger et al. 2016). A large meta-analysis by Wilson et al. (2006) found only marginal effects of
552 microcystins on zooplankton survival in general. This documented tolerance to *Microcystis*
553 by the Green Bay population could indicate that there has been strong selection for this
554 tolerance since the relatively recent invasion of the system a little more than 50 years ago.
555 However, it is also possible that tolerance to the effects of *Microcystis* is more general than
556 expected and already existed in the invasive clade before it invaded the Great Lakes. The
557 close similarity and evolutionary origin of nodularin and microcystin also supports this
558 possibility (Mur et al., 1999). Further studies comparing differences among *Eurytemora*
559 clades in tolerance to toxic cyanobacteria would be useful, as would studies of the evolution
560 of tolerance within populations.
561 *Reproductive output*
562 In the present study, female *Eurytemora* produced eggs at approximately the same rate in
563 both study areas (~5 eggs/female/day; Fig. 1C and 2C), but no differences were detected
564 between treatments in either of the experiments. Our results are consistent with those of
565 Sopanen et al. (2008) who found no changes in egg production rates of *Eurytemora* in either
566 algal mixtures containing toxic *Prymnesium parvum*, or in cell-free filtrates. On the other
567 hand, Nejstgaard and Solberg (1996) reported that toxins excreted by *Prymnesium* decrease
568 egg production of a common copepod (*Acartia clausi*), which did not prey upon *Prymnesium*.
569 The most plausible reason for *Eurytemora* continuing to produce eggs during low quality
570 food conditions is that females allocated energy reserves to offspring production during low

571 or unfavorable feeding conditions, which could have long-term consequences for their
572 condition or survival (as perhaps was observed in the Baltic FILT treatment). Ger and co-
573 authors (2009) show that *E. affinis* egg production was not affected by the ingestion of
574 *Microcystis*, but was highly dependent on the abundance of high quality food available (in
575 that case *Cryptomonas*) for sustaining egg production rates. Such findings suggest animals
576 can exhibit changes in energy allocation depending on feeding conditions.
577 *Trade-offs in egg production*
578 In the data presented here, nauplius length was negatively correlated with female egg
579 production rate for both populations examined, with the strongest effects observed when
580 animals were fed the mixed diets (Fig. 3, CYAN treatments). A reasonable body of evidence
581 supports a trade-off between offspring size and number, where increasing offspring quality is
582 constrained by the number of offspring produced (Roff, 1992), which our findings support.
583 Size is commonly considered a good indicator of offspring quality in ecological studies (Roff,
584 1992). In our experiment with animals from the Baltic Sea, nauplius size differed
585 significantly between treatments, decreasing when animals were fed either the mixture of
586 'good food' and cyanobacteria, or 'good food' and cyanobacteria filtrate. These differences
587 indicate negative maternal effects of the cyanobacteria treatment on offspring size because
588 lengths were determined for offspring in the first naupliar stage, which is a non-feeding stage.
589 Contrary to the current study, Vehmaa et al. (2013) revealed a positive trade-off between egg
590 quantity and quality for *Acartia* in the Baltic Sea; she and her coauthors showed that females
591 were able to allocate more resources to eggs when feeding on mixtures of good food and 20%
592 *Nodularia*, increasing both quality and viability of eggs. However, the good food source in
593 that experiment (*Brachiomonas*) was of lower nutritional quality than the *Rhodomonas*
594 employed in our experiments, and *Nodularia* therefore became an important nutritional
595 supplement.

In the Green Bay experiment, nauplius size was also negatively related to number of eggs produced, but only significantly so for the CYAN treatment (Fig. 3B). This relationship was observed even though there were no significant effects on ingestion rates, fecundity, or mean nauplius size for this treatment, possibly indicating the independent nature of this trait from the others measured. It is interesting that this same negative relationship was obtained even though Green Bay *E. carolleeae* on average produced smaller offspring than the *Eurytemora* sp. from the Baltic Sea. The largest nauplii produced in Green Bay are barely the size of the smallest nauplii from the Baltic Sea. This size difference is consistent with adult size differences between the two populations. Females from Green Bay were significantly smaller (mean prosome length 0.78 mm) than females from the Gulf of Finland (on average 0.95 mm). It is unclear what causes these size differences, but temperature has been shown generally to have a strong negative effect on copepod body size (Brun et al., 2016; Horne et al., 2016), and specifically on *E. affinis* in the Baltic Sea (Viitasalo et al., 1995). Brun et al. (2016) show that productivity and size selective predation have more complex relationships with copepod body size, varying both between species and on a local scale. Further research on factors affecting body size and other traits important for survival of copepods in the Great Lakes would be fruitful in light of these findings.

*Conclusion*

To conclude, despite using what are now considered different species of *Eurytemora* and different local cyanobacteria, both populations responded similarly to the food manipulations in terms of feeding. There were significant negative effects on ingestion rates of the filtrate derived from cyanobacteria cultures, and those effects were reduced when animals were fed a mixed diet. Both populations also exhibited some degree of tolerance to toxic cyanobacteria as survivorship and egg production were unaffected when animals were fed the mixed diets.

In addition, a negative relationship between nauplius length and fecundity of females was documented for both groups. However, effects on survivorship and nauplius size were different between the populations tested. Baltic *Eurytemora* sp. survival was significantly reduced in the cyanobacteria filtrate treatment, and smaller nauplii were produced, likely a result of a maternal effect due to reduced ingestion by mothers and/or lower allocation to egg production. These traits of Green Bay animals were not significantly affected by food treatments.

Tolerance to abiotic factors may facilitate dispersal success (Dodson et al., 2010; Hirsch et al., 2016), suggesting that tolerance to cyanobacteria could also promote the invasion of *Eurytemora* to new ecosystems, as seen in Lake Michigan. Lee et al. (2013) showed that high food availability and tolerance to lower salinity also promote invasions by *Eurytemora* to new systems. In Green Bay, *Eurytemora* may be forced to remain in dense blooms because the system is eutrophic and shallower (De Stasio et al., 2008), whereas in the Gulf of Finland, zooplankton can escape blooms by migrating deep (Almén et al., 2014). The use of lab-cultured food sources may limit the generality of our results, and caution must be exercised in drawing conclusions about how these two populations would respond to a more natural assemblage of food resources. Even given these limitations, our results are consistent with the conclusion that *E. carolleeae* in Green Bay may be more resistant to blooms and cyanobacteria toxicity than *Eurytemora* sp. from the Baltic Sea. The Green Bay population likely has undergone local adaptation following selection based on a number of novel conditions during its introduction and subsequent persistence in the Laurentian Great Lakes. Their tolerance to the cyanobacteria tested indicates the potential for future population expansion as blooms increase in the Great Lakes, or for successful secondary dispersal to inland eutrophic lakes where blooms occur.

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875 Table 1. Details of experiments on effects of toxic cyanobacteria on *Eurytemora*. Animals
876 were collected from the Baltic Sea in August 2012 or Green Bay, Lake Michigan in October
877 2013. Both experiments included treatments with good food (GF), good food plus
878 cyanobacteria (CYAN), or good food plus a volume of filtrate from cyanobacteria cultures
879 equivalent to the volume of cyanobacteria culture added (FILT). Baltic animals were fed
880 *Rhodomonas salina* (Rhod) and *Nodularia spumigena* (Nod) whereas Green Bay animals
881 were fed *Scenedesmus quadricauda* (Scen) and *Microcystis aeruginosa* (Mic). Mean
882 extracellular toxin concentration was measured as microcystin-LR equivalents (1 SE in
883 parentheses). Lower limit of toxin detection was 0.16 µg/L. Results of Tukey-Kramer
884 multiple comparison tests of toxin concentrations are indicated; values followed by the same
885 letter are not significantly different at the $p$=0.05 level.

886

| | | | Treatment | | |
|---|---|---|---|---|---|
| Location | Date | Treatment Conditions | GF | CYAN | FILT |
| Baltic Sea | Aug 2012 | Rhod (µg C/L) | 500 | 500 | 500 |
| | | Nod (µg C/L) | 0 | 100 | 0 |
| | | Nod Filtrate (equiv. vol.; µg C/L) | --- | --- | 100 |
| | | Replicates (n) | 3 | 3 | 3 |
| | | Toxin (µg/L) | <0.16 | 0.75 (0.06)a | 0.77 (0.06)a |
| Green Bay | Oct 2013 | Scen (µg C/L) | 500 | 500 | 500 |
| | | Mic (µg C/L) | 0 | 50 | 0 |
| | | Mic Filtrate (equiv. vol.; µg C/L) | --- | --- | 50 |
| | | Replicates (n) | 3 | 3 | 3 |
| | | Toxin (µg/L) | <0.16 | 0.52 (0.02)b | 0.80 (0.04)a |

887

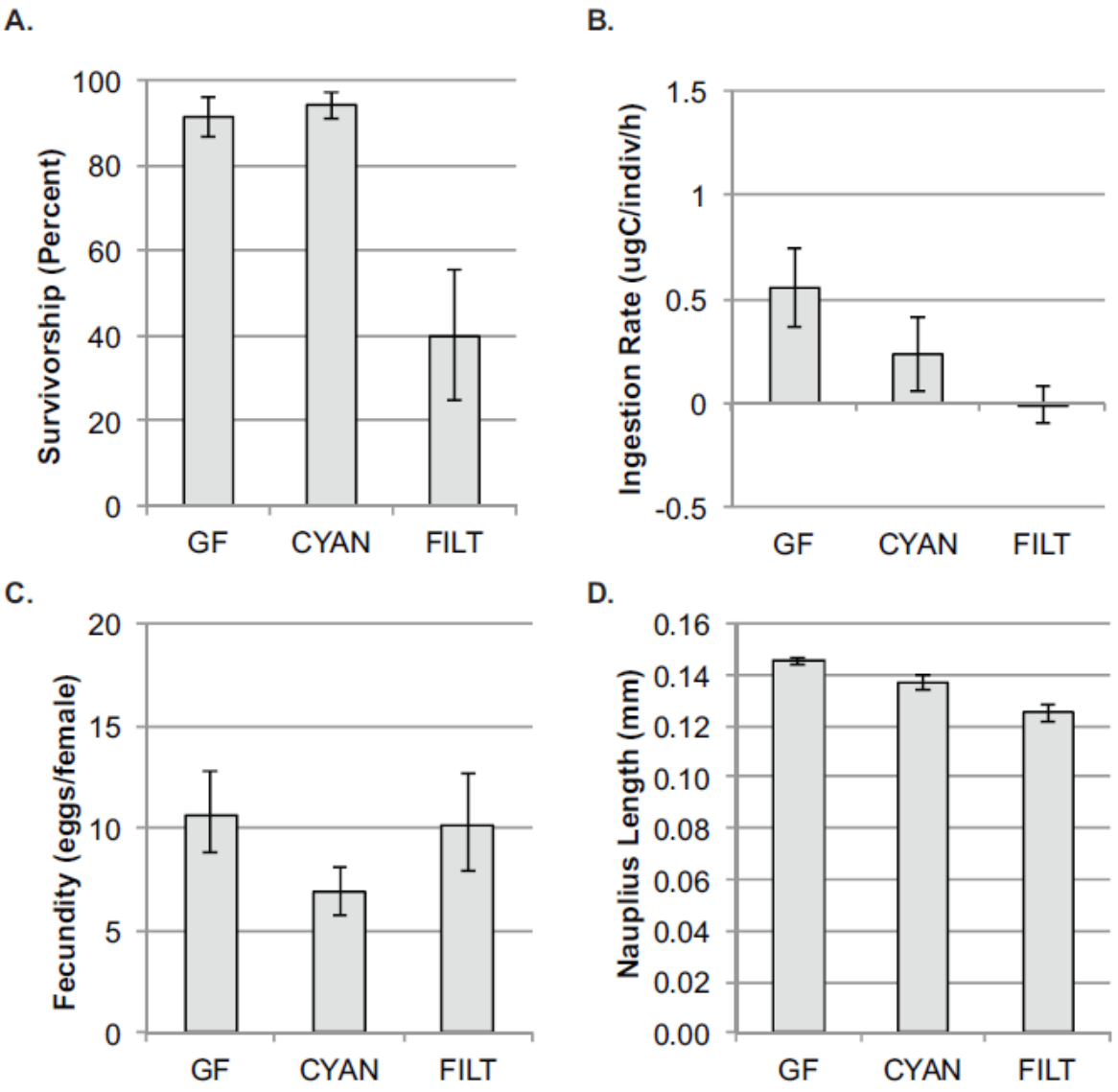
**Figure Captions**

Figure 1. Baltic Sea *Eurytemora* feeding experiment results for animals fed *Rhodomonas* (GF), *Rhodomonas* and *Nodularia* (CYAN) or *Rhodomonas* with filtrate from *Nodularia* cultures (FILT) during 2012. A) Mean percent survivorship of animals during experiment, B) Mean ingestion rates, C) Mean fecundity as eggs produced/female/day, and D) Mean nauplius length. Error bars represent ±1 standard error of the mean.

Figure 2. Green Bay *Eurytemora* feeding experiment results for animals fed *Scenedesmus* (GF), *Scenedesmus* and *Microcystis* (CYAN) or *Scenedesmus* with filtrate from *Microcystis* cultures (FILT) during 2013. A) Mean percent survivorship of animals during experiment, B) Mean ingestion rates, C) Mean fecundity as eggs produced/female/day, and D) Mean nauplius length. Error bars represent ±1 standard error of the mean.

Figure 3. Relationships between nauplius length (mm) and fecundity (eggs produced/female/day) for *Eurytemora* in A) Baltic Sea experiment in 2012 and B) Green Bay experiment in 2013. Separate regressions are plotted for animals in good food (GF), good food and cyanobacteria (CYAN), and good food with cyanobacteria filtrate (FILT) treatments. Note differences in nauplius length axes between experiments. See text for regression statistics.

908 Fig. 1.

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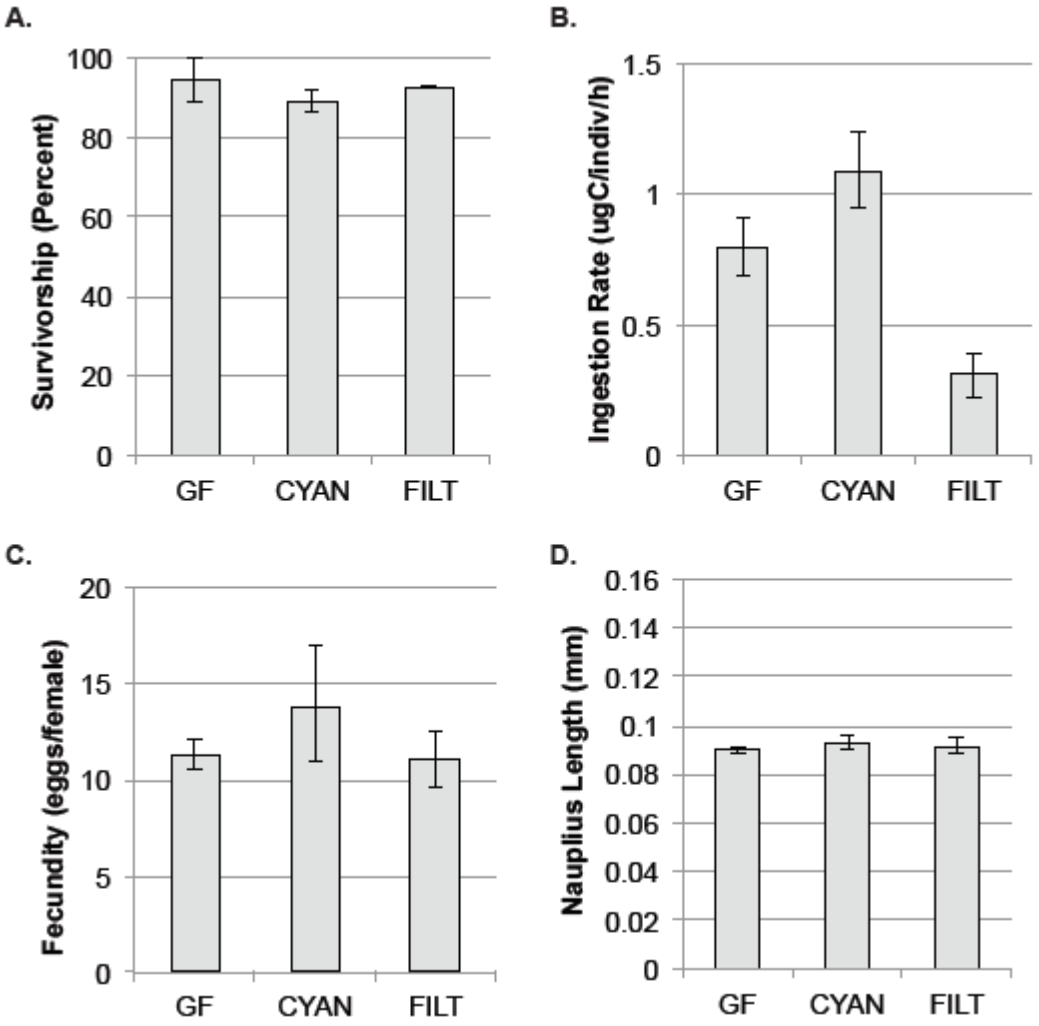
911 Fig. 2.

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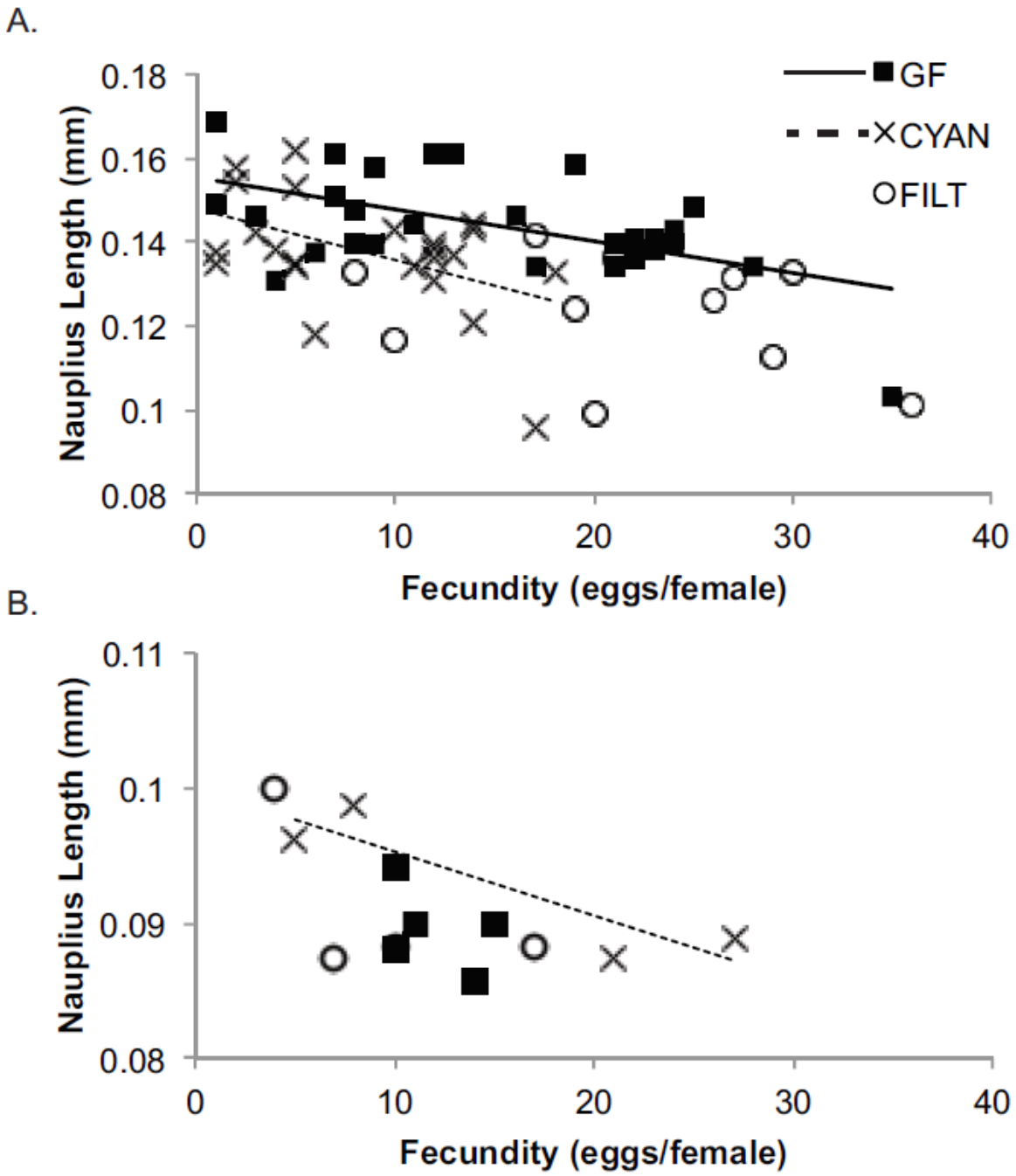
914 Fig. 3.

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